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*Science*. Author manuscript; available in PMC 2014 February 22.

Published in final edited form as:
*Science*. 2013 February 22; 339(6122): 975–978. doi:10.1126/science.1230751.

# Caspase-11 protects against bacteria that escape the vacuole

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## Abstract

Caspases are either apoptotic or inflammatory. The inflammatory Caspases-1 and -11 trigger pyroptosis, a form of programmed cell death. Whereas both can be detrimental in inflammatory disease, only Caspase-1 has an established protective role during infection. Herein, we report that Caspase-11 is required for innate immunity to cytosolic, but not vacuolar, bacteria. While *Salmonella typhimurium* and *Legionella pneumophila* normally reside in the vacuole, specific mutants (*sifA* and *sdhA*, respectively) that aberrantly enter the cytosol triggered Caspase-11, enhancing clearance of *S. typhimurium sifA in vivo*. This response did not require NLRP3, NLRC4, or ASC inflammasome pathways. *Burkholderia* species that naturally invade the cytosol also triggered Caspase-11, protecting mice from lethal challenge with *B. thailandensis* and *B. pseudomallei*. Thus, Caspase-11 is critical for surviving exposure to ubiquitous environmental pathogens.

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Canonical inflammasomes, such as NLRP3, NLRC4, and AIM2, are cytosolic sensors that detect pathogens or danger signals and activate Caspase-1, leading to secretion of the proinflammatory cytokines interleukin (IL)-1β and IL-18, and pyroptosis, a form of programmed cell death (1). Pyrin domain-containing inflammasomes, including NLRP3, signal through the ASC adaptor protein to recruit Caspase-1 (Fig. S1). Many diverse agonists cause cytosolic perturbations that are detected through NLRP3; however the underlying mechanisms remain obscure (2). In contrast, the CARD domain-containing inflammasome NLRC4 can signal directly to Caspase-1 resulting in pyroptosis, as well as indirectly through ASC to promote IL-1β and IL-18 secretion (Fig. S1) (1, 3). NLRC4 detects bacterial flagellin and type III secretion system (T3SS) rod or needle components within the macrophage cytosol (4–6). Together, NLRC4 and the ASC dependent inflammasomes account for all known canonical Caspase-1 activation pathways.

*Burkholderia pseudomallei* is a Gram-negative bacterium endemic to Southeast Asia that causes mellioidosis and is a potential biologic weapon (7). *B. pseudomallei* uses a T3SS to escape the phagosome and replicate in the cytosol. NLRC4 and NLRP3 both detect *B.*

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*pseudomallei*, promoting IL-1β secretion from murine bone marrow-derived macrophages (BMM) ((8) and Fig. 1A). Despite encoding many of the same virulence factors as *B. pseudomallei*, including T3SS and T6SS, the closely related *B. thailandensis* is far less virulent (9). We therefore hypothesized that NLPR3 and NLRC4 also detect *B. thailandensis*, and indeed, NLRP3 and NLRC4 accounted for all IL-1β secretion in response to *B. thailandensis* (Fig. 1B). We next determined whether inflammasome activation is critical to survival following *B. thailandensis* challenge using Caspase-1 deficient mice. Kayagaki et al. recently showed that all existing Caspase-1 deficient mice also lack Caspase-11 due to the backcrossing of a mutant *Casp11* allele from 129 into C57BL/6 mice (10). Inflammasome detection was critical for resistance to *B. thailandensis*, as *Casp1*$^{-/-}$*Casp11*$^{-/-}$ animals succumbed to the infection (Fig. 1C, S2A). In contrast, wild type C57BL/6 mice survived high dose intraperitoneal or intranasal challenge (Fig. 1C, S2A). Surprisingly, *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ mice that are deficient in all known canonical inflammasomes were also resistant (Fig. 1D, S2B). This indicated that an unknown signaling pathway provides protection via either Caspase-1 or -11 (see pathway schematic Fig. S1). Resistance to *B. thailandensis* was at least partially independent of IL-1β and IL-18, depending on the route of infection (Fig. 1E, S2C), suggesting that both cytokines and pyroptosis can contribute to protection. We therefore examined pyroptosis *in vitro*, and found that cytotoxicity in response *B. thailandensis* was impaired in *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMM (Fig. 1F). Consistent with our *in vivo* data, pyroptosis *in vitro* did not require *Nlrc4* or *Asc* (Fig. 1F). *B. pseudomallei* similarly triggered pyroptosis in *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ macrophages (Fig. 1G). These results indicate that a pyroptosis-inducing pathway distinct from all known canonical inflammasomes detects *B. thailandensis* and protects against lethal infection.

Inflammasomes discriminate pathogens from non-pathogens by detecting contamination or perturbation of the cytosolic compartment (11). The *B. thailandensis* T3SS facilitates bacterial access to the cytosol, and was required for induction of pyroptosis, whereas the virulence-associated T6SS was dispensable (Fig. 2A). We therefore hypothesized that macrophages detect vacuolar lysis or release of bacteria into the cytosol.

In order to establish their intracellular vacuolar growth niche, *Salmonella typhimurium* and *Legionella pneumophila* use T3SS and T4SS, respectively, to translocate effector proteins that work in concert to maintain the stability of these altered bacteria-containing vacuoles (12–14). Loss of the *S. typhimurium* SifA or *L. pneumophila* SdhA effectors causes rupture of the vacuole and release of bacteria into the cytosol (15–17). *S. typhimurium* uses two distinct T3SS encoded by the *Salmonella* pathogenicity island 1 (SPI1) and SPI2; these two T3SS translocate distinct batteries of effectors, such as SifA by SPI2 (18). While *S. typhimurium*-expressing SPI1 and flagellin are readily detected by NLRC4 (19, 20), bacteria grown under conditions that mimic the vacuolar environment express SPI2 and repress flagellin, minimizing canonical inflammasome detection (1, 11, 21). Infection of BMMs with *S. typhimurium* that lacked *sifA*, however, significantly increased IL-1β secretion and pyroptosis (Fig. 2B–C). IL-1β secretion was dependent on canonical inflammasomes (Fig. 2B), whereas pyroptosis was still observed in *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$*Nlrc4*$^{-/-}$ macrophages (Fig. 2C). Furthermore, the NLRC4 inflammasome agonist flagellin was not required for these responses (Fig. 2D, 2E). Thus, macrophages detect *S. typhimurium* when it aberrantly enters the cytosol, activating pyroptosis independent of all known canonical inflammasomes.

*L. pneumophila* also translocates flagellin through its T4SS. Thus, *L. pneumophila* mutants lacking flagellin (Δ*flaA*) evaded NLRC4 detection (Fig. 2F) (2). In contrast, *L. pneumophila* Δ*flaA* Δ*sdhA* mutants induce Caspase-1 activation (16, 17), IL-1β secretion (17), and pyroptosis (Fig. 2F; (17)). The AIM2-ASC canonical inflammasome has been implicated in

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*L. pneumophila* Δ*flaA* Δ*sdhA*-induced IL-1β secretion, likely by detecting DNA released from bacteria lysing in the cytosol; however, the role of AIM2-ASC in pyroptosis was not examined (17). Analogous to *S. typhimurium* Δ*sifA, L. pneumophila* Δ*flaA* Δ*sdhA* induced pyroptosis in the absence of flagellin and ASC (Fig. 2G), ruling out all canonical inflammasomes in triggering pyroptosis under these infection conditions. These data demonstrate that diverse bacteria are detected in the cytosol.

Because IL-1β secretion required the canonical inflammasomes whereas pyroptosis did not, we hypothesized that cell death is triggered by a distinct mechanism mediated by Caspase-11. Like Caspase-1, Caspase-11 is an inflammatory caspase that can directly trigger pyroptosis (Fig. S1). Caspase-11 can also promote IL-1β secretion dependent upon NLRP3, ASC, and Caspase-1 (10, 22–24). Because Caspase-1 is activated by recruitment to an oligomerized platform known as the inflammasome, Kayagaki et al. hypothesized that a similar oligomeric structure would activate Caspase-11, which they termed the non-canonical inflammasome (10). Although the cholera toxin B subunit and many different Gram-negative bacteria can trigger Caspase-11 activation *in vitro* (10, 22–24), the nature of the physiologic stimulus that activates Caspase-11 during infection remains uncertain.

Caspase-11 activation requires priming through a Toll like receptor 4 (TLR4)-TRIF-STAT1 pathway (10, 22–24). Consistent with this, *Tlr4*$^{-/-}$ and *Trif*$^{-/-}$ macrophages did not undergo pyroptosis after *S. typhimurium* Δ*sifA* infection, whereas cell death was observed in macrophages deficient in the other TLR4 adaptor, Myd88 (Fig. 3A). This dependence could be overcome by priming the macrophages with interferon (IFN)-γ (Fig. 3A), which signals through STAT1. Interestingly, IFN-γ or LPS priming significantly increased the sensitivity of macrophages to *S. typhimurium* Δ*sifA* (Fig. 3A, S3A). These priming effects correlated with increased Caspase-11 expression (Fig. S3B–C), but could also be mediated by enhancing aberrant vacuolar rupture. We used retroviruses to complement *Casp1*$^{-/-}$*Casp11*$^{-/-}$ macrophages with either *Casp1* or *Casp11* in order to determine which was involved. Caspase-11 alone promoted pyroptosis without IL-1β secretion after *B. thailandensis* infection, whereas Caspase-1 enabled both responses (Fig. 3B). This is consistent with *B. thailandensis* detection through NLRC4 and/or NLRP3 activating Caspase-1 (8) and an additional pathway activating Caspase-11. In contrast, the responses to *S. typhimurium* Δ*sifA* or *L. pneumophila* Δ*flaA* Δ*sdhA* acted through Caspase-11, and not Caspase-1 (Fig. 3C–D). We further confirmed that Caspase-11 was responsible for the cell death observed in *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ macrophages using short hairpin (sh)RNAmir (Fig. 3E–F, S3E). Finally, *Casp11*$^{-/-}$ BMM revealed that Caspase-11 was required for pyroptosis after *B. thailandensis, S. typhimurium* Δ*sifA*, and *L. pneumophila* Δ*flaA* Δ*sdhA* (Fig. 3G–I). Although a previous report suggested that NLRC4 signals through Caspase-11 to alter phagosomal trafficking (25), we saw no evidence that NLRC4 contributes to Caspase-11 dependent cell death (Fig. 1F, 2D, S4). Pyroptosis initiated by Caspase-11 was morphologically similar to pyroptosis triggered by Caspase-1 (Fig. S5A–B). Therefore, macrophages activate Caspase-11 in response to cytosolic *B. thailandensis, S. typhimurium*, or *L. pneumophila* (Fig. S1).

*S. typhimurium* Δ*sifA* is attenuated (15), attributed to the role of SifA in coordinating intracellular trafficking of the *Salmonella*-containing vacuole. We hypothesized that this attenuation was actually due to innate immune detection though Caspase-11. Indeed, *S. typhimurium* Δ*sifA* was mildly attenuated in C57BL/6 mice as expected, but this was not replicated in *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice (Fig. 4A–B). We next determined the relative clearance of *S. typhimurium* Δ*sifA* during co-infection with wild type *S. typhimurium*, a more quantitative measure of virulence than lethal challenge. 16-fold fewer *S. typhimurium* Δ*sifA* were recovered from C57BL/6 mice 48h post infection. However, only a 4-fold reduction was seen in *Casp11*$^{-/-}$ mice (Fig. 4C), indicating that Caspase-11 clears *S.*

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*typhimurium* Δ*sifA in vivo*; in contrast, wild type *S. typhimurium* effectively evades Caspase-11 (23) by remaining within the vacuole. The remaining *S. typhimurium* Δ*sifA* attenuation likely reflects the role of *sifA* as a virulence factor promoting intracellular replication. Moreover, all known canonical inflammasomes were dispensable for *S. typhimurium* Δ*sifA* clearance, as were IL-1β and IL-18 (Fig. 4D), implicating pyroptosis as the mechanism of clearance. Clearance of bacteria after pyroptosis is mediated by neutrophils through generation of reactive oxygen (21). Consistent with this, NADPH oxidase deficient *p47phox*$^{-/-}$ mice were also defective for clearance of *S. typhimurium* Δ*sifA* (Fig. 4D). Interestingly, TLR4 and IFN-γ were not required (Fig. 4E), suggesting that there is redundant priming of Caspase-11 pathways *in vivo*. Therefore, Caspase-11 protects mice from *S. typhimurium* Δ*sifA*, and because IL-1β and IL-18 are not required, pyroptosis is likely to be the mechanism of bacterial clearance in this case.

We next examined the susceptibility of *Casp11*$^{-/-}$ mice to the naturally cytosolic pathogens *B. thailandensis* and *B. pseudomallei*. While C57BL/6 mice are resistant to *B. thailandensis* infection, *Casp11*$^{-/-}$ mice succumbed (Fig. 4F). Likewise, *Casp11*$^{-/-}$ succumbed to *B. pseudomallei* infection, whereas C57BL/6 mice survived (Fig. 4G). Since *Nlrc4*$^{-/-}$ mice are also susceptible to *B. pseudomallei* infection (8), we conclude that both Caspase-1 and Caspase-11 play critical roles in limiting *B. pseudomallei* infection.

Collectively, these data demonstrate, for the first time, that Caspase-11 protects animals from lethal infection by bacteria that have the ability to invade the cytosol. This could be critical for defense against ubiquitous environmental bacteria such as *B. thailandensis* that encode virulence factors, but have not evolved to evade Caspase-11 detection. It will be interesting to determine whether Caspase-11 is activated in response to the process of vacuolar rupture or the presence of bacteria within the cytosol. Caspase-11 also responds to vacuolar bacteria under delayed kinetics, but such responses have not been shown to provide protection from infection *in vivo* (10, 22–24). LPS-induced septic shock is mediated by Caspase-11 (10), suggesting that Caspase-11 can be activated by other mechanisms besides cytosol-localized bacteria. Thus, we propose that Caspase-11 provides protection against pathogens, but is dysregulated during overwhelming infection, contributing to septic shock and mortality. It will be interesting to determine if Caspase-11 triggers eicosanoid secretion as is seen for Caspase-1, and whether these mediators contribute to septic shock (26). The identity of the hypothesized non-canonical inflammasome(s) that activate Caspase-11 and the precise nature of the activating signal will shed more light on the mechanisms by which Caspase-11 can both promote innate immunity and exacerbate immunopathology. These insights may lead to novel therapies to treat infection and sepsis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

The authors thank V. Dixit sharing mice (under a materials transfer agreement) and M. Heise for sharing mice, and S. Miller, J. Mougous, and H. Schweizer for sharing bacterial strains. We also thank D. Rodriguez and L. Zhou for managing mouse colonies. The data presented in this manuscript are tabulated in the main paper and in the supplementary materials. This work was supported by NIH grants AI097518 (EAM) and AI057141 (EAM and AA), AI065359 (PAC), AI075039 (REV), AI080749 (REV), and AI063302 (REV), Investigator Awards from the Burroughs Wellcome Fund and Cancer Research Institute (REV), and an NSF graduate fellowship (MFF).

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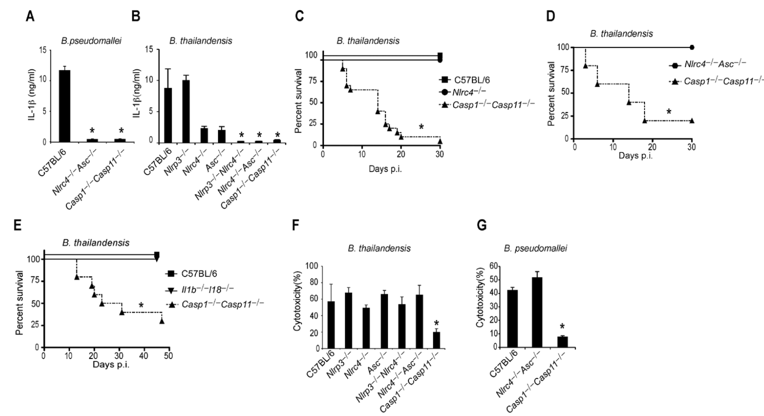


**Fig. 1. *Burkholderia* detection and protection conferred by *Casp1/11* is independent of all known canonical inflammasomes**

Lipopolysaccharide (LPS) primed BMMs were infected with *B. pseudomallei* (**A**, **G**) or *B. thailandensis* (**B**, **F**) for 4h. (**A**, **B**) IL-1β secretion was determined by ELISA or (**F**, **G**) cytotoxicity was determined by LDH release assay. (**C**, **D**, **E**) Survival curves of wild type C57BL/6 or the indicated knockout mice infected $2 \times 10^7$ cfu i.p. with *B. thailandensis*. Data are representative of at least 3 (**A**, **B**, **F**, **G**) or 2 (**D**, **E**) experiments. (**C**) Data are pooled from 3 experiments. For number of mice in each panel see Table S2. Statistically significant differences with respect to controls are indicated (Student's T-test or log rank test for survival; * = $p$ 0.05, n.s. = p > 0.05).



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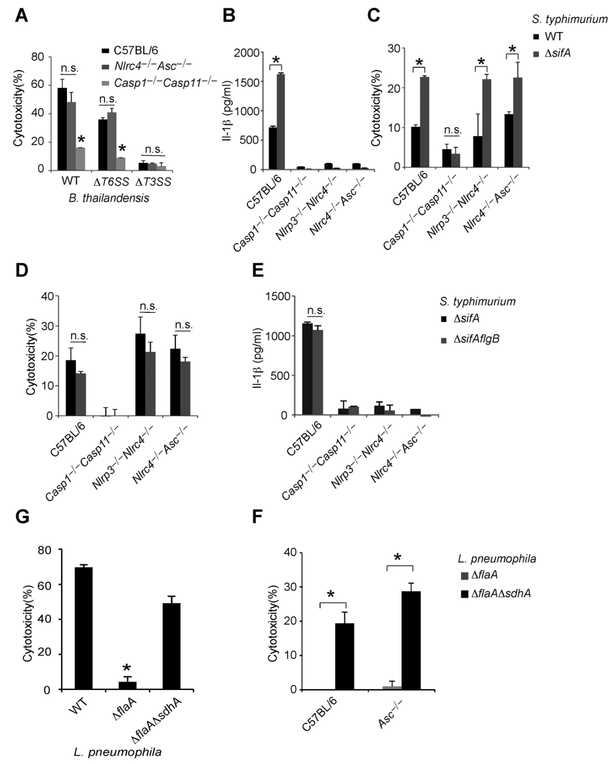

**Fig. 2. Diverse cytosolic bacteria activate pyroptosis independent of NLRC4, NLRP3 and ASC**
(**A**) LPS-primed BMMs were infected for 4h with either *B. thailandensis* or the indicated mutants and cytotoxicity was determined. BMMs were infected for 8h with (**B**) *S. typhimurium* or *S. typhimurium* Δ*sifA*, (**D**) *S. typhimurium* Δ*sifA* or *S. typhimurium* Δ*sifA flgB* (**D**) and cytotoxicity was determined. LPS-primed BMM were infected for 8h with *S. typhimurium* or *S. typhimurium* Δ*sifA* (**C**), *S. typhimurium* Δ*sifA or S. typhimurium* Δ*sifA flgB* (**E**) and IL-1β secretion was determined. (**F**, **G**) Cytotoxicity in wild type or $Asc^{-/-}$ BMMs infected for 4h with *L. pneumophila*, *L. pneumophila* Δ*flaA* or *L. pneumophila* Δ*sdhA* Δ*flaA*. Cytotoxicity was determined by LDH release and IL-1β secretion by ELISA. Data are representative of at least 3 experiments. Statistically significant differences with respect to controls are indicated (Student's T-test; * = $p$ 0.05, n.s. = p > 0.05).



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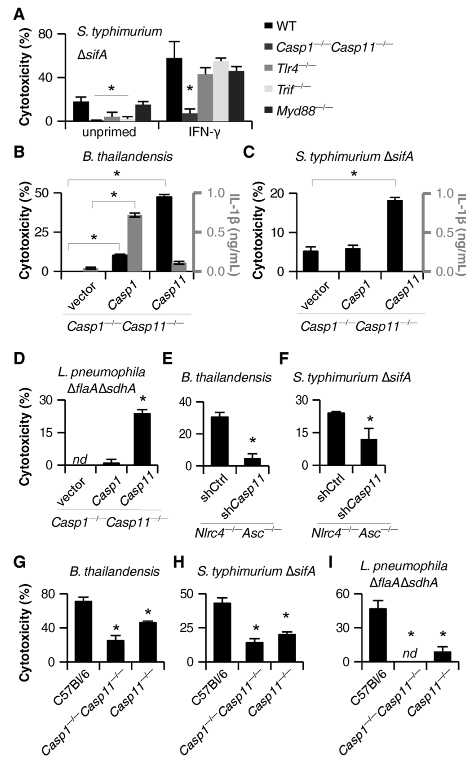


**Fig. 3. Caspase-11 mediates pyroptosis after infection by cytosolic bacteria. (A–I)** Macrophage cytotoxicity and IL-1β secretion were determined after infection with *S. typhimurium* Δ*sifA* (8h), *L. pneumophila* Δ*flaA* Δ*sdhA* (4h), or *B. thailandensis* (4h). (**A**) C57BL/6, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *Tlr4*$^{-/-}$, *Trif*$^{-/-}$, and *Myd88*$^{-/-}$ BMM infected with *S. typhimurium* Δ*sifA* with or without IFN-γ priming prior to infection. (**B–C**) Retroviral transduction was used to complement *Casp1* or *Casp11* in *Casp1*$^{-/-}$*Casp11*$^{-/-}$ iBMM. Macrophages were primed with LPS (**B**) or IFN-γ (**C**) and responses to *B. thailandensis* (**B**) or *S. typhimurium* Δ*sifA* (**C**) infection were examined. (**D**) Control or complemented *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMM infected with *L. pneumophila* Δ*flaA* Δ*sdhA*. (**E–F**) Retroviral transduction was used to introduce control or *Casp11*-targeting shRNAmir into *Nlrc4*$^{-/-}$*Asc*$^{-/-}$ iBMM. Macrophages were primed overnight with LPS (**E**) or IFN-γ (**F**) and then infected as indicated. (**G–I**) C57BL/6, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, and *Casp11*$^{-/-}$ BMM infected with *B. thailandensis* (**G**), *S. typhimurium* Δ*sifA* (**H**), or *L. pneumophila* Δ*flaA* Δ*sdhA* (**I**). Data are representative of at least 3 (**A–C**, **E**, **G**, **H**) or 2 (**D**, **F**, **I**) independent experiments. Statistically significant differences with respect to controls are indicated (Student's T-test; * = $p$   0.05). *nd*, none detected.

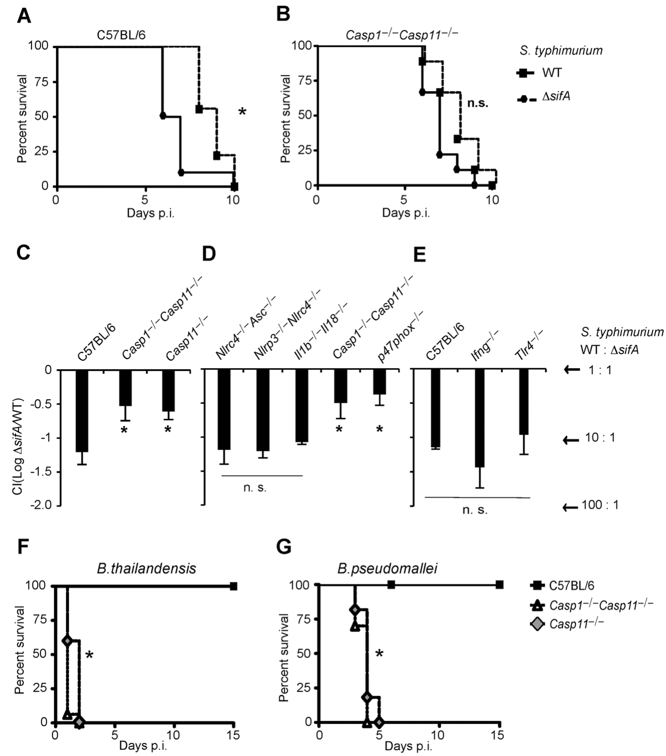


**Fig. 4. Caspase-11 protects against cytosolic bacteria *in vivo***

(**A**, **B**) *S. typhimurium* or *S. typhimurium* Δ*sifA* were injected i.p. into C57BL/6 (1000 cfu) or *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice (250 cfu) and survival was monitored. (**C–E**) The indicated mice were infected with $5\times10^4$ cfu of both wild type *S. typhimurium* and *S. typhimurium* Δ*sifA* marked with ampicillin or kanamycin resistance, respectively. Bacterial loads from 3–4 mice per genotype were determined 48h later and competitive index calculated (CI = log (*S. typhimurium* Δ*sifA*/ *S. typhimurium*)). A CI of −1 corresponds to 10 cfu of *S. typhimurium* for every 1 cfu of *S. typhimurium* Δ*sifA*. (**F–G**) C57BL/6, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, or *Casp11*$^{-/-}$ mice were infected with (**F**) $2\times10^7$ cfu mouse passaged *B. thailandensis* i.p. or (**G**) 100 cfu *B. pseudomallei* i.n. (**A**, **B**, **F**, **G**) Data are pooled from two independent experiments. (**C–E**) Representative of 2 experiments. For number of mice in each panel see Table S2. Statistically significant differences with respect to controls are indicated (Student's T-test or log rank test for survival; * = $p$ 0.05, n.s. = $p > 0.05$).



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